EXHIBIT A

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Differential Actions of Neurotrophins in the Locus Coeruleus and Basal Forebrain

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The neurotrophin gene family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/NT-5, supports the survival of distinct peripheral neurons, however, actions upon central neurons are relatively undefined. In this study we have compared different neurotrophins in the regulation of neuronal survival and function using dissociated embryonic cell cultures from two brain regions, the basal forebrain (BF) and locus coeruleus (LC). In the BF, NGF increased choline acetyl transferase (ChAT) activity, but did not influence cholinergic cell survival. In contrast to NGF, BDNF, NT-3, and the novel neurotrophin, NT-4, all increased ChAT activity and cholinergic cell survival. We also examined embryonic LC neurons in culture. LC neurons are unresponsive to NGF. In contrast, NT-3 and NT-4 elicited significant increases in survival of noradrenergic LC neurons, the first demonstration of trophic effects in this critical brain region. Identification of factors supporting coeruleal and basal forebrain neuronal survival may provide insight into mechanisms mediating degeneration of these disparate structures in clinical disorders. © 1993 Academic Press, Inc.

INTRODUCTION

Factors which regulate neuronal survival may be of critical importance for normal development and mature function of the central nervous system (2). A variety of data suggests that target-derived factors play a critical role in the survival of afferent neurons. The neurotrophin family of neurotrophic factors, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4, are all expressed in the hippocampus in the adult and at various stages of development. In the present studies we characterize the influence of the different neurotrophins on two afferent populations, the basal forebrain cholinergic neurons and the locus coeruleus noradrenergic population.

The most extensively characterized neurotrophic factor, NGF, has been shown to influence cholinergic function of basal forebrain (BF) neurons by increasing activity of choline acetyltransferase (ChAT), the acetylcholine-synthesizing enzyme (13, 31, 41). Further, infusion of NGF after fimbria–fornix transection prevents cholinergic cell death (16, 15, 33, 43), indicating that NGF may influence neuronal survival after a lesion. Damage itself may induce responsivity to NGF in adult basal forebrain neurons; cholinergic neurons are influenced by NGF after fimbria transection, but not in unlesioned adult rats (42, 16) or after removal of the hippocampal target without transection of the pathway (39). Thus, the role of NGF in supporting cholinergic cell survival in the normal, unlesioned brain is unclear. Responsivity of BF cholinergic neurons to NGF may depend on the physiological state of the neurons, either during a particular developmental period or after trauma.

NGF is now known to be a member of a neurotrophin gene family consisting of BDNF (28), NT-3 (19, 30, 7, 38), NT-4 (14), and NT-5 (3). The last appears to represent the mammalian homologue of NT-4 (23), first isolated from *Xenopus laevis* (14). BDNF and NT-3 are expressed in the hippocampus in overlapping but distinct patterns (9, 35). Moreover, BDNF influences BF cholinergic neurons and may support cholinergic innervation to the hippocampus (1, 26). NT-3-responsive neuronal populations remain undefined, although trophic effects have been observed in the BF (1). Expression of this factor in the brain is restricted to the hippocampus and certain cortical regions (11, 9, 35). The novel neurotrophin, NT-4/NT-5, is expressed at low levels in the adult rat brain (3), particularly in cerebral cortex and hippocampus (unpublished results), although potential functions in the mammalian brain are unknown.

We have examined the influence of specific neurotrophins on neuronal survival in the basal forebrain and locus coeruleus. Characterization of trophic requirements may provide insight into mechanisms mediating neuronal survival in these two brain regions which project to a common target.

METHODS

Brain Cultures

Time-pregnant Sprague–Dawley rats (Taconic Laboratories) were housed in clear plastic cages with *ad lib.*

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access to Purina Lab Chow and water. Animals were exposed to fluorescent illumination between 5 AM and 7 PM daily. Embryonic age was calculated from the day of discovery of the vaginal plug, which was Embryonic Day 1 (E1).

At E16 pregnant rats were sacrificed by exposure to CO_2 and soaked in 80% ethanol for 10 min. Fetuses were removed under sterile conditions and kept in PBS on ice for microscopic dissection of basal forebrain or the rostral rhombencephalon including the locus coeruleus (6). The tissue was dissociated by trituration and plated on polylysine-coated tissue culture dishes at a density of 1 million cells per 35-mm dish (Corning). The cells were maintained for 4 or 7 days in either serum-containing medium (Eagle's MEM, 7.5% FCS, 6 g/l glucose, penicillin–streptomycin), which promotes survival and proliferation of non-neuronal support cells, or in a serum-free medium which yields a relatively neuron-pure culture. The serum-free medium was composed of MEM and Ham's F-12 (1:1), glucose (6 mg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), selenium (30 n*M*), putrescine (60 μ*M*), progesterone (20 n*M*), and penicillin–streptomycin (0.5 U/ml–0.5 μg/ml). Concentrated serum-free COS media containing the different trophic factors were diluted to appropriate concentrations and added to the cells at the time of plating. Cultures were grown for 4 or 7 days without changing media or supplementing the factors.

Catalytic Assays

Basal forebrain cultures were assayed for changes in ChAT activity by measuring incorporation of [^{14}C]-choline into [^{14}C]acetylcholine (10). Briefly, cells were harvested in 10 m*M* EDTA with 0.5% Triton and incubated with sodium phosphate (50 m*M*, pH 7.4), EDTA (20 m*M*), NaCl (300 m*M*), choline bromide (8 m*M*), physostigmine (0.1 m*M*), and [^{14}C]acetyl-CoA (0.2 m*M*) for 1 hr at 37°C. The assay was stopped with the addition of 10 m*M* sodium phosphate (pH 7.4) and 2 ml acetonitrile with 10 mg tetraphenylboron and counted in a liquid scintillation counter (Beckman) using a toluene scintillant.

Histochemical Procedures

(a) For acetylcholinesterase histochemistry, cultures were incubated in a 50 m*M* acetate buffer with 4 m*M* acetylthiocholine, 2 m*M* copper sulfate, 10 m*M* glycine, and 0.2 m*M* ethopropazine to inhibit nonspecific esterases. Cultures were then rinsed, exposed to 1.25% Na_2S, rinsed again, and exposed to 1% $AgNO_3$ (17). Labeled cells were counted in 2.5% of the area of the dish using a Zeiss Axiovert microscope.

(b) For tyrosine hydroxylase (TH) immunocytochemistry, cultures were fixed in 10% formalin and exposed to anti-TH antiserum. Cells were visualized using the avidin–biotin technique for immunoperoxidase staining. After incubation with biotinylated anti-rabbit IgG, cultures were exposed to the ABC reagent (Vector Laboratories). Cells were visualized by the 3,3-diaminobenzidine reaction product. All labeled cells in the dish were counted.

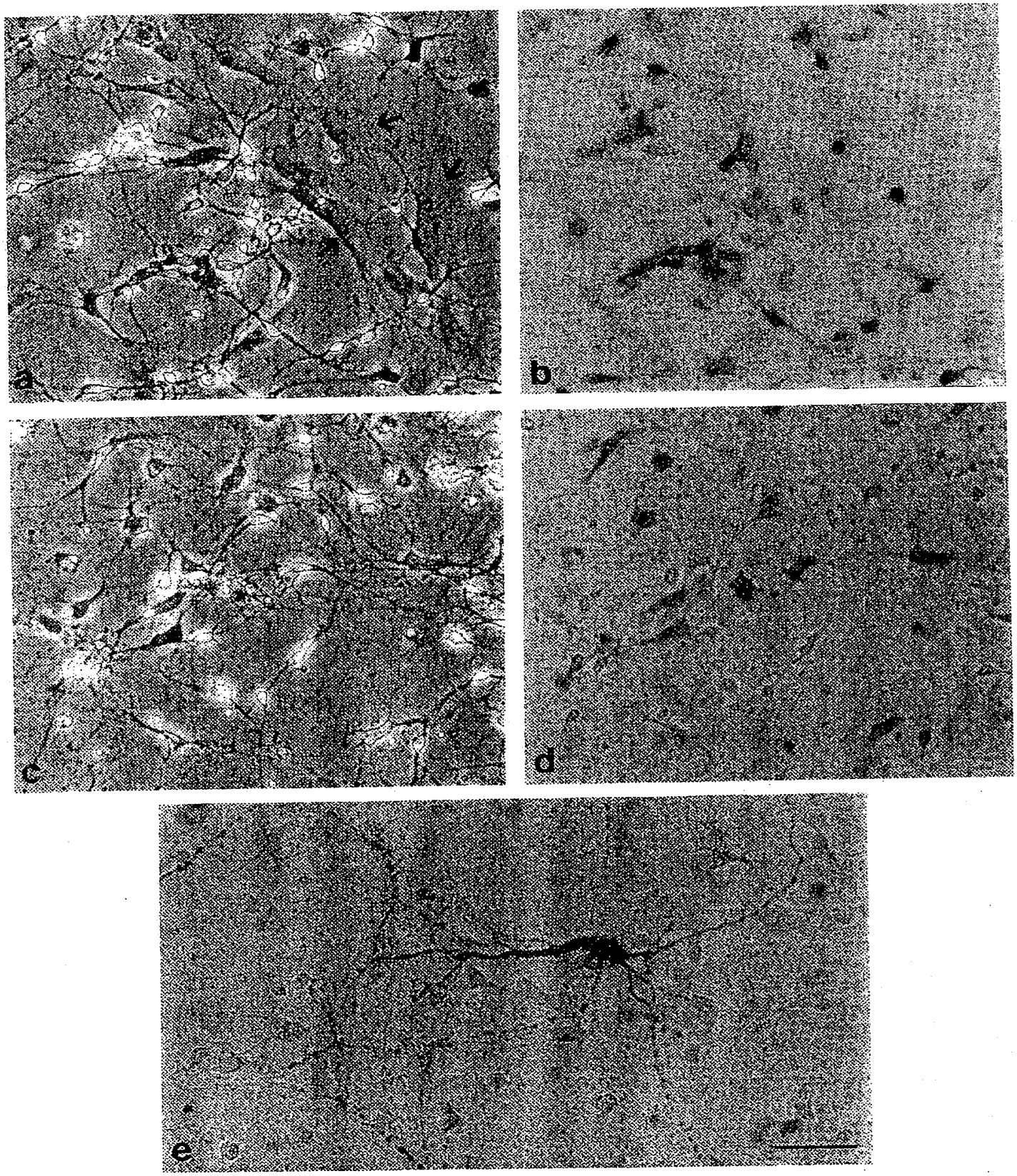
Production of Neurotrophins

The protein coding regions of the sequences for rat NGF, BDNF, NT-3, and *Xenopus* NT-4 were cloned into the expression vector pXM, a plasmid which is suitable for transient expression in COS cells (21). These cells were transfected with plasmids using the DEAE-dextran/chloroquine technique (29). Parallel transfections with an unrelated plasmid containing the β-galactosidase gene served as control. One day after transfection the medium was changed to a serum-free medium, and the cells were allowed to grow for 3 days. At this time the medium conditioned with the factors produced and secreted by the transfected COS cells was collected and concentrated 100× in Amicon concentrating tubes such that proteins larger than 10 kDa were retained. COS cells transfected with the control plasmid were assayed for β-galactosidase activity to assess the efficiency of transfection (20). The amount of trophic factor protein produced was assessed by growing parallel plates in the presence of [^{35}S]cysteine (22). The labeled conditioned COS media was then analyzed by SDS–polyacrylamide gel electrophoresis. The gels were treated with EnHance (NEN), dried, and subjected to autoradiography. Autoradiograms were analyzed using a Shimadzu densitometer. The amount of trophic factor produced was assessed by the amount of novel protein appearing in the transfected media migrating at approximately 13 kDa. Further, the amount of bioactive trophic factor produced by the COS cells was assessed by bioassay on peripheral ganglia and estimation of biological units in induction of neurite outgrowth (7). COS cell transfection routinely yielded in the range of 100 ng/ml of NGF and NT-4, and 20–50 ng/ml of BDNF and NT-3.

RESULTS

Neurotrophic Influences on the Locus Coeruleus

Trophic support of noradrenergic neuronal survival was monitored in the locus coeruleus (LC). Dissociated LC cultures from E16 rat fetuses were grown in serum with concentrated COS media containing 10–50 ng/ml of the different neurotrophins, or with concentrated media from cells transfected with a control plasmid. Cells were labeled immunocytochemically with antiserum directed against tyrosine hydroxylase (Fig. 1e). The number of TH-positive cells surviving after 1 week in culture increased twofold in the presence of NT-3 or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NT-4 (Fig. 2), providing the first demonstration of neurotrophic influences in the locus coeruleus.

Cholinergic Cell Survival in Basal Forebrain Cultures

To determine whether cholinergic neuron survival was influenced by the different neurotrophins, E16 basal forebrain dissociated cell cultures were grown with NGF, BDNF, NT-3, or NT-4. Cholinergic neurons were labeled by acetylcholinesterase histochemistry (Figs. 1a–1d). The addition of 10–50 ng/ml of COS cell-produced BDNF, NT-3, or NT-4 elicited significant increases in cholinergic cell number (Fig. 3), suggesting a possible role in supporting neuronal survival. In contrast, NGF did not alter the number of cholinergic neurons in BF cultures. To ascertain whether COS cell medium may have masked an effect, 100 ng of purified 2.5S NGF was compared to COS NGF. Neither NGF preparation influenced cholinergic cell number (Fig. 3).

To assess whether COS cell-produced NGF had biological activity in the BF cultures, effects on ChAT activity were examined. COS NGF elicited a 2-3-fold increase in ChAT activity (Fig. 4), confirming previous results with purified NGF, and suggesting that this neurotrophin influences cholinergic function, but not neuronal survival in developing basal forebrain neurons in culture.

Influence of Neurotrophins in Pure Neuronal BF Cultures

BDNF, NT-3, and NT-4 influenced cholinergic neurons in BF cultures grown in the presence of serum as above. To ascertain whether the effects of these neurotrophins was exerted directly on the cholinergic neurons, cultures were grown under serum-free conditions, yielding a virtually pure neuronal population (Figs. 1c–1d). These cultures contained fewer than 5% glia. Using this preparation, BDNF, NT-3, and NT-4 elicited significant twofold increases in ChAT activity (Fig. 5), suggesting that the neurotrophins acted directly on the neuronal population.

DISCUSSION

In these studies, we have found that noradrenergic neurons of the locus coeruleus, which are insensitive to NGF (5, 34), respond to other neurotrophins, significantly increasing noradrenergic cell survival. Survival of LC neurons is known to be increased by hippocampal neurons in culture (37), suggesting that the target elaborates trophic factors. Specifically, both NT-3 (7, 9, 35), and NT-4 (unpublished data) are expressed in the hippocampus and appear to provide trophic support for LC neurons. The trophic actions of NT-3 in the locus coeruleus may be mediated by interactions with trkC, a receptor specific for NT-3 (27), which is expressed in the LC in adult rats (32). In contrast, NT-4 has been shown to activate trkB (23), suggesting that this receptor may be expressed in the LC as well.

Our studies also indicated that BDNF, NT-3, and NT-4 fostered BF cholinergic neuronal survival and function. We were able to dissociate effects on neurotransmitter parameters from those on cell survival. NGF, though eliciting increases in ChAT activity, failed to influence cholinergic cell survival, in contrast to the effects of BDNF, NT-3, and NT-4. The role of NGF in mediating cholinergic function but not survival indicates that the different neurotrophins may be highly specialized in their mechanisms of action. The complex trophic interactions require the presence of appropriate receptors on responsive neurons. The low affinity NGF receptor, p75, which binds all the neurotrophins, has been localized to the basal forebrain (44, 12), as have high affinity binding sites (4). The trk protooncogene product, which binds NGF (24, 18), and trkB, which binds BDNF (25, 40) and NT-4 (23), are both expressed in the adult rat basal forebrain (32). Moreover, trk mRNA has been detected in the E18 rat BF (8).

Both the locus coeruleus and basal forebrain innervate the hippocampus, a rich source of neurotrophic factors. Neurotrophin-3, which is synthesized in the hippocampus (9, 35), supported survival of both BF cholinergic neurons and LC noradrenergic neurons, indicating that a mutual target may synthesize a common trophic factor to support distinct neuronal pathways. Although recent data has demonstrated the presence of neurotrophins in the local environment of some responsive neurons, the absence of BDNF and NT-3 from the basal forebrain (11, 12) and locus coeruleus (8) suggests a possible target-derived mode of action with the hippocampus as the source of trophic support.

NT-4 also significantly increased both noradrenergic and cholinergic survival, as well as influencing ChAT activity. The trophic effects of NT-4 reported in this study were seen using recombinant NT-4 from *X. laevis*. In bioassays on peripheral sensory neurons from dorsal root and nodose ganglia, *Xenopus* and rat NT-4 have identical bioactivity (14, 23). Moreover, *Xenopus* and rat NT-4 are indistinguishable in receptor activation, eliciting specific tyrosine phosphorylation of trkB (23, and unpublished results). Thus, the *Xenopus* NT-4 is

FIG. 1. Dissociated cultures from the E16 embryonic rat brain. Basal forebrain neurons grown in either serum (a, b) or serum-free (c, d) conditions were labeled by acetylcholinesterase histochemistry (a–d). Cultures are shown using phase-contrast (a, c) or bright-field (b, d) microscopy. Arrows in (a) indicate flat support cells. Note absence of flat cells in c. A noradrenergic neuron from the locus coeruleus (e) was labeled immunocytochemically using anti-TH antiserum. Size bar in e is 50 μm and is the same for all panels.

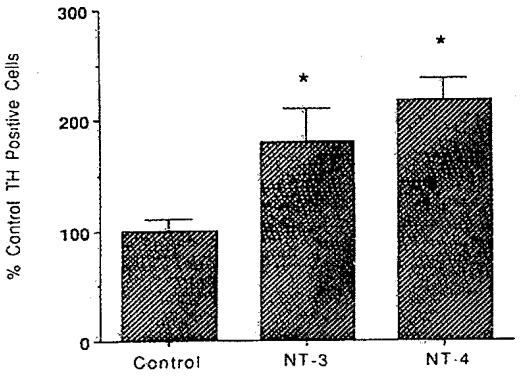

FIG. 2. Effects of NT-3 and NT-4 on TH neuron survival. Dissociated locus coeruleus cultures were grown in serum-containing media in the presence of COS-produced NT-3, NT-4, or control. TH-positive cells were counted and analyzed by analysis of variance. Data are shown as mean percentage control ± SEM from four independent experiments. Control LC cultures contained 121 ± 18 TH-positive neurons per dish. $^*P < 0.05$.

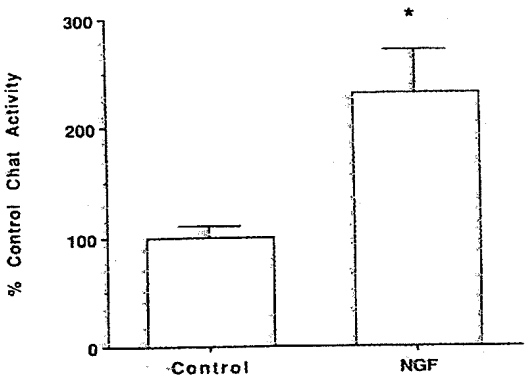

FIG. 4. Effect of COS-produced NGF on cholinergic function. ChAT activity was assayed in E16 BF dissociated cultures grown in serum and exposed to COS NGF or control in 10 independent experiments. $^*P < 0.05$.

capable of activating the rat trkB receptor, supporting the results obtained in the present study. Our observations on the trophic effects of NT-4 raise intriguing possibilities concerning the newly discovered NT-5, which appears to be the human homologue of *Xenopus*/viper NT-4 (3, 23). NT-5 has been detected in the adult rat brain (3), particularly in the cortex (unpublished results), another target common to both the basal forebrain and locus coeruleus.

The recombinant neurotrophins used in this study were derived from conditioned media from transfected COS cells without further biochemical purification. Similar amounts of the different neurotrophins were used in each assay, as assessed by quantification of autoradiograms from SDS–polyacrylamide gels of media from metabolically labeled COS cells. COS-produced NGF and purified 2.5S NGF elicited identical trophic responses in basal forebrain, indicating that the preparations have specific trophic activity. Moreover, no effects were seen with conditioned media from mock-transfected cells.

In sum, we have defined neurotrophic influences in two critical brain regions, the basal forebrain and locus coeruleus. Specific neurotrophins such as NT-3 and NT-4 enhanced survival of both neuronal populations. NGF, which has no effect on LC neurons, influenced the BF by increasing cholinergic function but not survival, in contrast to BDNF, NT-3, and NT-4. The discovery that neuronal survival in both the basal forebrain and locus coeruleus is affected by common target-derived trophic factors provides a possible mechanism by which

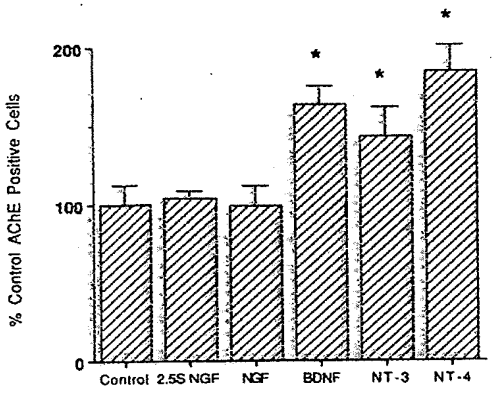

FIG. 3. Influence of neurotrophins on cholinergic neuron survival. Basal forebrain dissociated cell cultures were grown in serum-containing media in the presence of either control COS media, COS-produced neurotrophins, or purified 2.5S NGF. Acetylcholinesterase-positive cells were counted in three independent experiments and analyzed by ANOVA. Data are expressed as the mean percentage control ± SEM. $^*P < 0.05$.

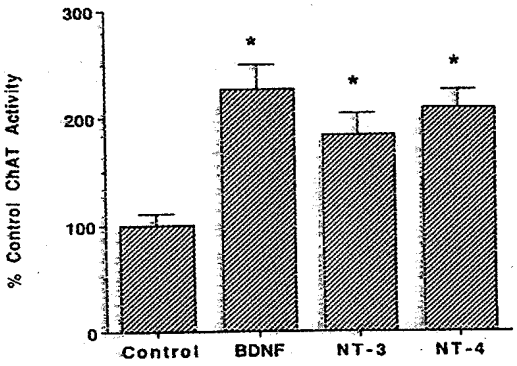

FIG. 5. Effects of neurotrophins in pure neuronal BF cultures. ChAT activity was assayed in basal forebrain dissociated cultures grown under serum-free conditions in the presence of COS control, BDNF, NT-3, or NT-4 concentrated media. Data are expressed as mean percentage control ± SEM from six independent experiments. $^*P < 0.05$.

these distinct populations undergo necrosis in a single degenerative disorder such as Alzheimer's disease.

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